More Sensitive Immune Complex Transfer Enzyme Immunoassay for Antibody IgG to p17 of HIV-1 With Shorter Incubation Time for Immunoreactions and Larger Volumes of Serum Samples

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In the previous immune complex transfer enzyme immunoassay for antibody IgG to p17 of HIV-1, the immune complex comprising 2,4-dinitrophenyl-bovine serum albumin-recombinant p17 conjugate, anti-p17 IgG, and recombinant p17- β -D-galactosidase conjugate was trapped onto polystyrene beads coated with (anti-2,4-dinitrophenyl group) IgG by overnight incubation and was transferred to polystyrene beads coated with (antihuman IgG γ -chain) IgG by 3 hr incubation in the presence of excess of ϵ N-2,4-dinitrophenyl-L-lysine. These processes were made efficient by incubation with shaking and

by using solid phases with larger surface areas. In addition, the volume of serum samples used was increased from 10 μ l to 100 μ l. As a result, the sensitivity was improved 20–30-fold and was $\sim$ 100,000-fold higher than that of Western blotting for p17 band, even when both trapping and transferring of the immune complex were performed for only 30 min. Furthermore, testing many samples became easily possible with higher sensitivity using microplates and a fluororeader. J. Clin. Lab. Anal. 11:244–250, 1997. © 1997 Wiley-Liss, Inc.

Key words: HIV-1; anti-HIV-1 IgG; enzyme immunoassay; β -D-galactosidase, fluororeader

INTRODUCTION: TESTING SENSITIVE IMMUNE COMPLEX TRANSFER ENZYME IMMUNOASSAYS

Ultrasensitive enzyme immunoassays (immune complex transfer enzyme immunoassays) for antibody IgGs to p17, p24 and reverse transcriptase (RT) of HIV-1 have been developed using recombinant p17, p24, and RT (rp17, rp24, and rRT) as antigens (1–7). The immune complex, comprising 2,4-dinitrophenyl-recombinant protein conjugate, antibody IgG to HIV-1, and recombinant protein- β -D-galactosidase conjugate, was trapped onto polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG by overnight incubation. After washing, the immune complex was eluted from the polystyrene beads with ϵ N-2,4-dinitrophenyl-L-lysine and was transferred to polystyrene beads coated with affinity-purified (antihuman IgG γ -chain) IgG by 3 hr incubation. Bound β -D-galactosidase activity was assayed by fluorometry. The volume of serum samples used was 10 μ l. These

immunoassays have made possible diagnosis of HIV-1 infection with urine (1–4) and whole saliva samples (5,6) and have been shown to be more useful as a confirmatory test for HIV-1 infection with higher sensitivities and specificities than Western blotting (7). Notably, antibody IgG to p17 of HIV-1 could be detected as early as or even earlier than antibodies to HIV-1 by conventional methods (7,8). In addition, an ultrasensitive enzyme immunoassay for p24 antigen of HIV-1 has been developed by applying the principle of immune complex transfer (9,10). Finally, the window period after HIV-1 infection, during which diagnosis of the infection is not possible due to the absence of detectable antibodies to HIV-

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1, has been shortened by simultaneous detection of both p24 antigen and antibody IgGs to p17 and RT (8). In these immunoassays, however, incubation time for immunoreactions was rather long (overnight incubation for trapping of the immune complex followed by 3 hr incubation for transferring of the immune complex).

This report describes more sensitive immune complex transfer enzyme immunoassays for antibody IgG to p17 of HIV-1 with shorter incubation time for immunoreactions and larger volumes of serum samples.

MATERIALS AND METHODS

Buffer

The regularly used buffer was 10 mmol/L sodium phosphate buffer, pH 7.0, containing 1.0 g/L bovine serum albumin (fraction V, Interger Co., Purchase, NY), 1.0 mmol/L $MgCl_2$, and 1.0 g/L NaN_3 (buffer A).

Recombinant p17 (rp17) of HIV-1

Recombinant p17 (rp17) was produced in *Escherichia coli* transformed with expression plasmid carrying the corresponding cDNA and was purified as described previously (1,11). The recombinant proviral clone used was pNL4-3 (12), which contained DNA from HIV-1 isolate NY5 (GenBank accession number HIVNL43) and LAV (13), and the sequence for p17 derived from NY5.

Solid Phases Coated With Affinity-Purified (anti-2,4-dinitrophenyl group) IgG

Solid phases were coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG by physical adsorption as described previously (14). The concentrations of antibody IgG used for coating were 50 μ g/ml for colored polystyrene beads with a diameter of 3.2 mm and white polystyrene beads with diameters of 5.0 and 6.3 mm (Immuno Chemical, Okayama, Japan) and 5, 10, and 25 μ g/ml for polystyrene sticks (Sumitomo Bakelite Co., Osaka, Japan) (Fig. 1) (15). After coating, solid phases were stored in buffer A containing 0.1 mol/L NaCl at 4°C until use.

Solid Phases Coated With Affinity-Purified (antihuman IgG γ -chain) IgG

White polystyrene beads with a diameter of 3.2 mm (Immuno Chemical) and black microplate wells (H type, Sumitomo Bakelite Co.) were coated with affinity-purified (antihuman IgG γ -chain) IgG by physical adsorption as described previously (14). There was no significant difference in test results by the previous and present immune complex transfer enzyme immunoassays using solid phases coated with different concentrations (5–100 μ g/ml) of affinity-purified (antihuman IgG γ -chain) IgG. After coating, the solid phases

Fig. 1. Polystyrene stick for coating with affinity-purified (anti-2,4-dinitrophenyl group) IgG (15).

were stored in buffer A containing 0.1 mol/L NaCl at 4°C until use.

Previous Immune Complex Transfer Enzyme Immunoassay (I)

Antibody IgG to p17 of HIV-1 was measured essentially in the same way as described previously (Fig. 2) (7). An aliquot (10 μ l) of serum samples was incubated for at least 0.5 hr with 140 μ l of buffer A containing 0.4 mol/L NaCl, inactive β -D-galactosidase (50 μ g), and 100 fmol each of 2,4-dinitrophenyl-bovine serum albumin-rp17 conjugate and rp17- β -D-galactosidase conjugate and was incubated for 1 hr or overnight with two colored polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG in a test tube. The two colored polystyrene beads after washing were incubated for 1 hr or 3 hr with two white polystyrene beads (3.2 mm in diameter) coated with affinity-purified (antihuman IgG γ -chain) IgG in a test tube containing 150 μ l of buffer A containing 0.1 mol/L NaCl and 1 mol/

Fig. 2. Immune complex transfer enzyme immunoassay for antibody IgG. DNP: 2,4-dinitrophenyl group. Ag: antigen. Enz: enzyme.

L ϵ N-2,4-dinitrophenyl-L-lysine. All the above incubations were performed at room temperature without shaking. After washing, β -D-galactosidase activity bound to the two white polystyrene beads was assayed for 1 hr at room temperature by fluorometry using 4-methylumbelliferyl- β -D-galactoside as substrate (16). The substrate solution (150 μ l) after enzyme reaction was mixed with 2.5 ml of 0.1 mol/L glycine-NaOH buffer, pH 10.3, and the fluorescence intensity was measured by adjusting that of 1×10^{-8} mol/L 4-methylumbelliferone to 100 using 360 nm for excitation and 450 nm for emission analysis with a spectrofluorophotometer (F-3010, Hitachi, Tokyo, Japan). In some experiments, the substrate solution after enzyme reaction was mixed with 50 μ l of a mixture of 0.1 mol/L glycine-NaOH buffer, pH 10.3 (50 μ l), and 8 mol/L NaOH (3 μ l), and the fluorescence intensity was measured with a fluororeader (Fluoroskan II, Labsystems, Helsinki, Finland) as described previously (15).

Previous Immune Complex Transfer Enzyme Immunoassay (II)

The previous immune complex transfer enzyme immunoassay (I) was modified as follows. An aliquot (10 μ l) of serum samples mixed with inactive β -D-galactosidase and the two conjugates was incubated for 1 hr with four colored polysty-

rene beads (3.2 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG in a microplate well (transparent, S type, Sumitomo Bakelite Co.). The polystyrene beads after washing were incubated for 1 hr with ϵ N-2,4-dinitrophenyl-L-lysine in a black microplate well coated with affinity-purified (antihuman IgG- γ -chain) IgG. All the incubations were performed at room temperature without shaking.

Previous Immune Complex Transfer Enzyme Immunoassays (III) and (IV)

The previous immune complex transfer enzyme immunoassay (I) was modified as follows. An aliquot (10 μ l) of serum samples mixed with inactive β -D-galactosidase and the two conjugates was incubated for 1 hr with one white polystyrene bead (5 mm (III) or 6.3 mm (IV) in diameter) in a microplate well (transparent, S type, Sumitomo Bakelite Co.). The white polystyrene bead after washing was incubated for 1 hr with ϵ N-2,4-dinitrophenyl-L-lysine in a black microplate well coated with affinity-purified (antihuman IgG- γ -chain) IgG. All the incubations were performed at room temperature without shaking.

Previous Immune Complex Transfer Enzyme Immunoassay (V)

The previous immune complex transfer enzyme immunoassay (I) was modified as follows. An aliquot (10 μ l) of serum samples mixed with inactive β -D-galactosidase and the two conjugates was incubated for 1 hr with one polystyrene stick (Fig. 1) (15) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG in a microplate well (transparent, S type, Sumitomo Bakelite Co.). The polystyrene stick after washing was incubated for 1 hr with ϵ N-2,4-dinitrophenyl-L-lysine in a black microplate well coated with affinity-purified (antihuman IgG- γ -chain) IgG. All the incubations were performed at room temperature without shaking.

Present Immune Complex Transfer Enzyme Immunoassays (I), (II), (III), (IV), and (V)

The present immune complex transfer enzyme immunoassays (I), (II), (III), (IV), and (V) were performed in the same way as the previous ones (I), (II), (III), (IV), and (V), respectively, described above except for the following modifications. The volumes of serum samples used were 10 and 100 μ l. When 100 μ l of serum samples was used, the concentration of NaCl in the final mixture of serum samples and the two conjugates was adjusted to 0.4 mol/L assuming that in serum to be 0.15 mol/L. The incubations were performed for 0.25–1 hr at room temperature with 180 shakings per min. Bound β -D-galactosidase activity was assayed for 1 hr at room temperature throughout.

Other Immunological Methods

The gelatin particle agglutination test for antibodies to HIV-1 was performed using a commercial kit with a lysate of HIV-1 as antigen (SERODIA-HIV, Fujirebio, Tokyo, Japan). Western blotting for antibody IgGs to HIV-1 was performed using a commercial kit preblotted with nine proteins of HIV-1 (gp160, gp120, p66, p55, p51, gp41, p31, p24, and p17) (Ortho HIV Western Blot Kit, Ortho Diagnostic Systems, Raritan, NJ).

Serum Samples From HIV-1 Seronegative and Seropositive Subjects

Serum samples were collected from an HIV-1 seropositive subject and 45 HIV-1 seronegative subjects (23 males aged 22–62 yr and 22 females aged 31–64 yr) and were stored at -20°C until use. The seronegativity was confirmed by the gelatin particle agglutination test (Fujirebio).

RESULTS AND DISCUSSION

The previous immune complex transfer enzyme immunoassays (Fig. 2) were performed as summarized in Table 1 (7,15). Antibody IgG to p17 was reacted with 2,4-dinitrophenyl-bovine serum albumin-rp17 conjugate and rp17- β -D-galactosidase conjugate for 3 hr. The immune complex formed was trapped onto solid phase coated with (anti-2,4-dinitrophenyl group) IgG and was transferred to solid phase coated with (antihuman IgG γ -chain) IgG by incubation with ϵ N-2,4-dinitrophenyl-L-lysine. In the previous immune complex transfer enzyme immunoassay (I), the immune complex formed was trapped onto two colored polystyrene beads with a diameter of 3.2 mm by overnight or 1 hr incubation and was transferred to two white polystyrene beads with a diameter of 3.2 mm by 3 hr or 1 hr incubation. In the previous immune complex transfer enzyme immunoassay (II), the immune complex formed was trapped onto four colored polystyrene beads with a diameter of 3.2 mm by 1 hr incubation and was transferred to a black microplate well by 1 hr incubation. In the previous immune complex transfer enzyme immunoassays (III) and (IV), the immune complex formed was trapped onto a polystyrene bead with diameters of 5.0 mm and 6.3 mm, respectively, by 1 hr incubation and was transferred to a black microplate well by 1 hr incubation. In the previous immune complex transfer enzyme immunoassay (V), the immune complex formed was trapped onto a polystyrene stick by 1 hr incubation and was transferred to a black microplate well by 1 hr incubation. The volume of serum samples used was 10 μl , and all the incubations were performed at room temperature without shaking throughout (Table 1). Signals (fluorescence intensities for bound β -D-galactosidase activity) were measured with a spectrofluorophotometer.

The present immune complex transfer enzyme immunoassays (I), (II), (III), (IV), and (V) were performed using the same solid phases as those in the previous immune complex transfer enzyme immunoassays (I), (II), (III), (IV), and (V), respectively, with the following modifications (Table 1). Antibody IgG to p17 was incubated with 2,4-dinitrophenyl-bovine serum albumin-rp17 conjugate and rp17- β -D-galactosidase conjugate for at least 30 min, since the formation of the immune complex was almost completed within 15–30 min. Trapping and transferring of the immune complex were performed for 0.25–1 hr by incubation with shaking throughout. The volume of serum samples used was 10 or 100 μl .

Enhancement of Signals by Increasing the Surface Area of Solid Phases

In the previous immune complex transfer enzyme immunoassay (I) using two polystyrene beads with a diameter of 3.2 mm, signals for serum from an HIV-1 seropositive subject by 1 hr trapping and 1 hr transferring of the immune complex were 21–24% of those by overnight trapping and 3 hr transferring of the immune complex (Table 1). In the previous immune complex transfer enzyme immunoassays (II), (III), (IV), and (V) by 1 hr trapping and 1 hr transferring of the immune complex using solid phases with larger surface areas, signals for serum from an HIV-1 seropositive subject increased to 41–110% of those by overnight trapping and 3 hr transferring of the immune complex (Table 1). Signals for serum from HIV-1 seronegative subjects were not significantly different among different solid phases.

Improvement of the Sensitivity by Shorter Incubation With Shaking for Immunoreactions

Signals for serum (10 μl) from an HIV-1 seropositive subject by the present immune complex transfer enzyme immunoassays, in which both trapping and transferring of the immune complex were performed by 0.5 hr incubation with shaking were 187–555% of those by overnight trapping and 3 hr transferring of the immune complex without shaking described above. In the present immune complex transfer enzyme immunoassay (V) using polystyrene sticks, signals for serum (10 μl) of an HIV-1 seropositive subject even by 0.25 hr trapping and 0.25 hr transferring of the immune complex were still higher than (175% of) those by the previous immune complex transfer enzyme immunoassay (I) with overnight trapping and 3 hr transferring of the immune complex. Signals for serum samples of HIV-1 seronegative subjects were similar in the present and previous immune complex transfer enzyme immunoassays. Thus the sensitivity of the immune complex transfer enzyme immunoassay was markedly improved by incubation with shaking for immunoreactions despite of considerably shortened incubation time.

As shown in Table 1, signals for serum from an HIV-1 seropositive subject by both the previous and present immune

TABLE 1. Comparison of Signals by Present and Previous Immune Complex Transfer Enzyme Immunoassays for Antibody IgG to p17 of HIV-1 (measured with spectrofluorophotometer)^a

Immune complex transfer enzyme immunoassay	Solid phase (diameter, surface area of beads or stick)	Volume of serum samples	Incubation time for		Signal (fluorescence intensity for bound β -D-galactosidase activity) with serum samples from		
			trapping the immune complex (hr)	transferring the immune complex (hr)	Sero-negative subject(s)	Seropositive subject	
						diluted 10 ⁵ -fold	diluted 10 ⁴ -fold
Previous, without shaking	(I) Two beads per tube (3.2 mm, 64 mm ²)	10	overnight	3	0.3 $\pm$ 0.1 (0.1 to 0.9)	12	103
	(II) Four beads per well (3.2 mm, 128 mm ²)	10	1	1	0.5	2.9	22
	(III) One bead per well (5.0 mm, 79 mm ²)	10	1	1	–	–	107
	(IV) One bead per well (6.3 mm, 125 mm ²)	10	1	1	–	–	113
	(V) One stick per well (~120 mm ²)	10	1	1	0.5	6.9	54
Present, with shaking	(I) Two beads per tube (3.2 mm, 64 mm ²)	10	0.5	0.5	0.0	20	193
		10	1	1	0.1 $\pm$ 0.1 (0.1 to 0.4)	38	377
		100	1	1	0.2 $\pm$ 0.3 (0.0 to 1.4)	310	–
	(II) Four beads per well (3.2 mm, 128 mm ²)	10	0.5	0.5	0.2	59	572
	(III) One bead per well (5.0 mm, 79 mm ²)	100	0.5	0.5	0.0	457	–
	(IV) One bead per well (6.3 mm, 125 mm ²)	10	0.5	0.5	–	–	448
	(V) One stick per well (~120 mm ²)	10	0.25	0.25	0.0	44	472
		10	0.5	0.5	0.2	22	180
		10	1	1	0.2	40	359
		100	0.5	0.5	0.5	53	465
		100	1	1	0.4	255	–
		100	1	1	0.2	403	–

^aSerum from an HIV-1 seropositive subject was diluted 10⁴- and 10⁵-fold with pooled serum from HIV-1 seronegative subjects. Bound β -D-galactosidase activity was assayed at room temperature for 1 hr. Some signals for HIV-1 seronegative subjects were expressed as mean $\pm$ SD and range in parentheses (n=50).

complex transfer enzyme immunoassays varied depending on surface areas of solid phases used except for those with polystyrene sticks. Signals with polystyrene sticks were lower than those with four polystyrene beads with a diameter of 3.2 mm and one polystyrene bead with a diameter of 5.0 or 6.3 mm, which had surface areas similar to or smaller than that of polystyrene sticks. There was no significant difference in signals with polystyrene sticks coated with different concentrations (5, 10, and 25 μ g/ml) of affinity-purified (anti-2,4-dinitrophenyl group) IgG. This might have been due to the fact that surface of polystyrene sticks was smooth but that of other solid phases were rough.

Further Improvement of the Sensitivity by Increasing the Volume of Serum Samples

When the present immune complex transfer enzyme immunoassays (II) and (V) were performed by 0.5 hr trapping and 0.5 hr transferring of the immune complex using 100 μ l of serum samples, signals for serum from an HIV-1 seropositive subject were 21–38-fold higher than that by the previous immune complex transfer enzyme immunoassay using 10 μ l of serum samples with overnight trapping and 3 hr transferring of the immune complex without shaking described above (Table 1). Signals for HIV-1 seronegative subjects were

TABLE 2. Comparison of Signals by Present and Previous Immune Complex Transfer Enzyme Immunoassays for Antibody IgG to p17 of HIV-1 (measured with fluororeader)^a

Immune complex transfer enzyme immunoassay	Solid phase (diameter, surface area of beads or stick)	Volume of serum samples	Incubation time for		Signal (fluorescence intensity for bound β -D-galactosidase activity) with serum samples from		
			trapping the immune complex (hr)	transferring the immune complex (hr)	Sero-negative subject(s)	Seropositive subject	
						diluted 10 ⁵ -fold	diluted 10 ⁴ -fold
Previous, (I) without shaking	Two beads per tube (3.2 mm, 64 mm ²)	10	1	1	0.048	0.25	2.1
Present, (I) with shaking	Two beads per tube (3.2 mm, 64 mm ²)	10	0.5	0.5	0.013	2.0	19
(V)	One stick per well (~120 mm ²)	10	0.5	0.5	0.078	4.8	43

^aSerum from an HIV-1 seropositive subject was diluted 10⁴- and 10⁵-fold with pooled serum from HIV-1 seronegative subjects. Bound β -D-galactosidase activity was assayed for 1 hr at room temperature.

not significantly changed. Thus the sensitivity of the present immune complex transfer enzyme immunoassays for antibody IgG to p17 using 100 μ l of serum samples was markedly (21–38-fold) improved as compared with that of the previous one and was ~100,000-fold higher than that of Western blotting for p17 band, even when both trapping and transferring of the immune complex were performed for 0.5 hr.

Quick Measurement of Signals With Fluororeader

In order to make easy testing many samples, signals were measured with a fluororeader (Table 2). Signals of 96 samples were obtained within 2 min. Signals by the present immune complex transfer enzyme immunoassays (I) using two polystyrene beads with a diameter of 3.2 mm and (V) using polystyrene sticks, in which both trapping and transferring of the immune complex were performed for 0.5 hr, were 10-fold and 20-fold, respectively, higher than those by the previous immune complex transfer enzyme immunoassay (I) using two polystyrene beads with a diameter of 3.2 mm, in which both trapping and transferring of the immune complex were performed for 1 hr. This was consistent with the results shown in Table 1, which were obtained with a spectrofluorophotometer.

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